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(54) Title: MONOCLONAL ANTIBODIES AGAINST TENASCIN

(57) Abstract

This invention provides monoclonal antibodies that bind to four non-overlapping epitopes on human tenascin. This invention also provides an improved method for determining metastatic potential of melanomas, based on the discovery that metastatic melanomas secrete tenascin.

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## + DESIGNATIONS OF "SU"

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# MONOCLONAL ANTIBODIES AGAINST TENASCIN

## BACKGROUND OF THE INVENTION

### Field of the Invention

This invention concerns monoclonal antibodies specific for human tenascin. This invention also concerns the use of monoclonal antibodies specifically reactive with human tenascin for diagnosing metastatic potential of melanoma and for determining the size of glioma tumors during surgery.

### Brief Description of the Prior Art

Tenascin, also known as hexabrachion, myotendinous antigen, glioma mesenchymal extracellular matrix antigen, J1 glycoprotein, cytotactin, GP-250 and gp 150/225, is one of the extracellular matrix proteins. Human tenascin is a high mass oligomeric glycoprotein with disulfide-bonded subunits of 320 kD. It has a hexametric structure with two pairs of three arms connected to a central globule.

Tenascin shows site-restricted expression during embryogenesis and can be found in adult tissues during wound healing and tumorigenesis. During embryonic development it is found in the condensing mesenchyme of developing organs such as mammary glands, hair follicles, toothbuds and kidneys (Chiquet-Ehrismann, et al., (1986), Cell, 47:131-139). For neural crest cells it lines the future migratory pathways (Crossin, et al., (1986), J. Cell Biol., 102:1917-1930). Tenascin is largely absent in normal adult tissue, but can be found during wound healing (Mackie, et al., (1988), J. Cell Biol., 107:2757-2767), and it is expressed in the stroma of a variety of solid tumors, including gliomas (Bourdon, et al., Cancer Res., 43:2796-2805, 1983), mammary (Chiquet-Ehrismann, et al., 1986) and lung carcinomas (Natali, et al. (1989), Intl. J. Cancer, 54:66-68),

fibrosarcomas and squamous cell carcinomas (Lightner, et al., (1989). J. Cell Biol., 108:2483-2493). The exact function of tenascin is not yet known, but it seems to play an important role in cell adhesion and spreading.

Polyclonal antisera to chicken tenascin have been reported, as well as a murine monoclonal antibody to human tenascin, designated 81C6 (Bourdon, et al., 1983). Monoclonal antibodies to chicken tenascin have also been prepared (Spring, et al., Cell, 59:325-334, 1989, and references therein). Using these various antibodies, tenascin has been located in the tissues, but it has not been reported in bodily fluids.

Imaging studies of tumors using radioactive-iodine-labelled anti-tenascin have been reported by Bourdon, et al. (Anticancer Res., 4:133-140, 1984), Bullard, et al. (J. Neurosurg., 64:257-262, 1986), and Blasberg, et al. (Cancer Res., 47:4432-4443, 1987) for human glioma tumor grafts in mice, and by Mackie, et al. (Proc. Natl. Acad. Sci. USA, 84:4621-4625,1987) for rat mammary tumors. Using monoclonal antibody 81C6, tenascin has been identified in a number of tumors: gliomas, breast carcinomas, neuroblastomas, melanomas, and various sarcomas (Bourdon, et al., 1983) and basal cell carcinomas (Stamp, J. Pathol., 159:225-229,1989). While tenascin was found in gliomas, it was not found in normal brain tissues. Mackie, et al. (1987), using polyclonal antisera to chicken tenascin, noted that tenascin could be found in rat mammary tumors but not in normal mammary tissue.

Melanomas are malignant tumors that develop from pigmented skin lesions. Four different cell types are associated with pigmented skin lesions, including dysplastic nevus, early primary melanoma (radial growth phase), late primary melanoma (vertical growth phase) and metastatic melanoma. Metastatic potential is associated with primary melanomas in vertical growth phase and metastatic melanomas. However, determining the metastatic potential of a lesion in vertical growth phase is difficult. Currently, the criteria for making this determination involves a number of factors, evaluated by an experienced diagnostician, who interprets the status of these factors in a particular case. The factors usually used

include: Thickness of the lesion, the mitotic rate of cells in the lesion, the level of infiltration by tumor infliterating lymphocytes (TIL), the location of the lesion on the patient, the sex of the patient and whether regression of the lesion is apparent. Additional factors which can be correlated with metastatic potential, and therefore could be used to provide confirmatory criteria which will reinforce the determination of metastatic potential, are needed, especially if the criteria can be quantitated.

## SUMMARY OF THE INVENTION

It is an object of this invention to provide monoclonal antibodies specific for epitopes on human tenascin.

It is also an object of this invention to provide an improved diagnostic method for the prediction of the metastatic potential of pigmented skin lesions.

It is another object of this invention to provide a rapid diagnostic method for determining the boundary of glioma tumors.

It is yet another object of this invention to provide an improved method for determining the optimum scope of tissue removal in surgical excision of glioma tumors.

This invention provides monoclonal antibodies that specifically bind to epitopes on human tenascin, wherein the antibody may also comprise a detectable label.

This invention further contemplates a method for detecting human tenascin, comprising: obtaining a sample of a human body fluid suspected of containing tenascin; contacting the sample with a monoclonal antibody specific for tenascin; and detecting immunocomplex.

This invention still further contemplates an improved method for determining metastatic potential of a pigmented skin lesion of a human patient, wherein the metastatic potential is judged by traditional criteria, including measuring the thickness of the lesion, measuring the mitotic rate within the lesion, and determining the number of tumor invading lymphocytes (TIL) within the lesion, the improvement comprising additionally determining the tenascin level

in serum from the patient, an elevated tenascin level indicating increased metastatic potential.

This invention also contemplates an improved method of therapy for glioma comprising surgical excision of the glioma tumor, wherein the improvement comprises preparing frozen sections from the boundary of the excised tissue; contacting the frozen sections with a monoclonal antibody specific for tenascin; and, if immunocomplex formation is detected, excising additional tissue.

By developing monoclonal antibodies from mice immunized with cells from multiple melanoma lines, the present invention provides monoclonal antibodies specific for a number of epitopes on the tenascin molecule that were previously unknown. It has also been discovered that metastatic melanomas secrete tenascin. Thus, patients with metastatic melanomas will have elevated levels of circulating tenascin, and a diagnostic test which detects this circulating tenascin can be used in evaluating whether metastatic melanoma is present in a given patient.

Tenascin is also produced by glioma tumors, where it can serve as a marker for tumor tissue because normal mature cells of the surrounding tissue do not produce tenascin. The present invention makes use of this phenomenon to develop a novel method of monitoring surgery to ensure that tumor tissue is excised, thus reducing the likelihood of remission.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Binding of mAb 300-3 to purified human extracellular matrix proteins in indirect solid phase RIA, as well as binding of control antibodies specific for either collagen, laminin, fibronectin, or tenascin.

Fig. 2. Quantitation of secretion of tenascin by primary and metastatic melanoma cells and normal melanocytes.

## DETAILED DESCRIPTION OF THE INVENTION

The term "antibody" as used in this invention is meant to include intact immunoglobulin molecules as well as fragments thereof, such as, for example, Fab and $F(ab')_2$, which are capable of binding the same epitopic determinant. As used in this invention, the

term "epitope" is meant to include any determinant capable of specific interaction with the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Monoclonal Antibodies

This invention provides monoclonal antibodies which specifically bind to epitopes on human tenascin. The monoclonal antibodies of this invention have specificity corresponding to that of one of the monoclonal antibodies produced by the hybridomas designated 300-1, 300-2, 300-3, 302-1 or 302-9.

Alternative Methods for Obtaining the Monoclonal Antibodies of the Invention

The present invention encompasses any monoclonal antibody that recognizes one of the epitopes recognized by antibodies from the hybridomas 300-1, 300-2, 300-3, 302-1 or 302-9. In another embodiment, the present invention contemplates monoclonal antibodies that correspond to the monoclonal antibody produced by hybridoma 300-1, 300-2 or 302-1, and, in a particularly preferred embodiment, the antibody produced by hybridoma 300-3 or 302-9. One antibody corresponds to another antibody if they both recognize the same or overlapping antigen binding sites as demonstrated by, for example, a binding inhibition or competitive binding assay.

Monoclonal antibodies according to this invention can be produced readily by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is now well known to the art. See, e.g., M. Schreier et al., Hybridoma Techniques (Cold Spring Harbor Laboratory 1980); Hammerling et al., Monoclonal Antibodies and T-Cell Hybridomas (Elsevier Biomedical Press 1981); Kennett et al., Monoclonal Antibodies (Plenum Press 1980). Generally, a rat or mouse is immunized with the antigen to be detected, and the rodent will later be sacrificed and spleen cells recovered for fusion with myeloma cells. Hybrid cells can be selected according to techniques known in the art. Antibody

production of each hybrid cell can be screened individually to select antibodies which have the specificity of the monoclonal antibodies of this invention.

Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier, et al., "Hybridoma Techniques" (1980); Hammerling, et al., "Monoclonal Antibodies and T-Cell Hybridomas" (1981); Kennett, et al., "Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917; 4,472,500; 4,491,632; 4,493,890.

The monoclonal antibodies produced by hybridomas 300-1, 300-2, 300-3, 302-1 or 302-9, can be readily employed to precipitate tenascin. For example, tenascin can be immunoprecipitated from tumor cell extracts. The precipitated antigen can be used as an immunogen, as can tenascin purified by other methods.

Tenascin, whether as a purified protein, as a component of serum, in tumor homogenates, or tumor cell culture, (e.g., mouse, rat, hamster, etc.), can be used as an immunogen to challenge the mammal to be used as a source of B-lymphocytes. The tenascin-stimulated B-lymphocytes are then harvested and fused to an immortal cell line or transformed into an immortal cell line by any appropriate technique. Antibody-producing immortal cells can be screened for production of the desired antibody by selecting clones that are strongly reactive with the antigen tenascin. Antibodies produced by clones which show those properties can then be screened for antibodies which correspond to antibodies produced by the deposited hybridomas.

The specificities of antibodies produced from cells of mammals immunized with tenascin can be determined readily through routine screening by one skilled in the art. Since the epitopic specificity of the monoclonal antibodies of the invention has been clearly defined, it is possible to greatly restrict the responder B-cell clones which are present for hybridoma fusion and thereby avoid undue experimentation in isolating hybridomas of the desired specificity.

By application of the above methods, one skilled in the art can readily produce a panel of monoclonal antibodies specifically binding the desired antigen.

The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the invention can be accomplished by one of ordinary skill in the art by the technique of anti-idiotypic screening (Potocnjak, et al., Science, 215: 1637, 1982). An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the monoclonal antibody produced by the hybridoma of interest. These determinants are located in hypervariable region of the antibody. It is this region which binds to a given epitope and, thus, is responsible for the specificity of the antibody.

The anti-idiotypic antibody can be prepared by immunizing an animal with the monoclonal antibody of interest. The animal immunized will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants. By using the anti-idiotypic antibodies of the second animal, which are specific for the monoclonal antibodies produced by a single hybridoma which was used to immunize the second animal, it is now possible to identify other clones with exactly the same idiotype as the antibody of the hybridoma used for immunization. Idiotypic identity between monoclonal antibodies of two hybridomas demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using antibodies to the epitopic determinants on a monoclonal antibody it is possible to identify other hybridomas expressing monoclonal antibodies of the same epitopic specificity.

Under certain circumstances, monoclonal antibodies of one isotype might be more preferable than those of another in terms of their diagnostic or therapeutic efficacy. Particular isotypes of a monoclonal antibody can be prepared either directly by selecting from the initial fusion, or prepared secondarily from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class-switch variants

(Steplewski, et al., Proceedings of the National Academy of Science, U.S.A., 82: 8653, 1985; Spira, et al., Journal of Immunological Methods, 74: 307, 1984). Thus, the monoclonal antibodies of the invention would include class-switch variants having the specificity of monoclonal antibody produced any of the hybridomas 300-1, 300-2, 300-3, 302-1, or 302-9. When the monoclonal antibodies of the invention are used in the form of fragments, such as, for example, Fab and $F(ab')_2$, and especially when these fragments are labeled, any isotype can be used.

The antibodies contemplated by this invention may optionally be derivatized with non-immunoglobulin moieties, so long as the added moiety does not affect the specificity of the antibodies. One class of moieties that may be added to the antibodies within the contemplation of this invention are detectable labels which enhance detectability of the antibody in, for instance, an immunoassay.

**Immunoassay Methods**

The monoclonal antibodies of the invention are particularly suited for use in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of tenascin using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal antibody,

or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the monoclonal antibody of the invention can be done using standard techniques commonly known to those of ordinary skill in the art.

Another technique, which may also result in greater sensitivity, consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts with avidin, or alternatively dinitrophenyl, pyridoxal, or fluorescein, which can react with specific anti-hapten antibodies.

The monoclonal antibodies of the invention can be bound to many different carriers and used to detect the presence of tenascin. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. Those skilled in the art will know of other suitable carriers for binding monoclonal antibody, or will be able to ascertain such, using routine experimentation.

For purposes of the invention, the tenascin which is detected by the monoclonal antibodies of the invention may be present in biological fluids or in tissues. Any sample containing a detectable amount tenascin can be used. Normally, a sample is a liquid such as urine, saliva, cerebrosphinal fluid, blood, serum and the like, or a solid or semi-solid such as tissues, feces, and the like.

The immunoassay procedures contemplated by this invention may use any antibody which is specific for human tenascin, including monoclonal antibody 81C6. The preferred antibodies for use in the immunoassays contemplated by this invention are those corresponding to the monoclonal antibodies produced by the hybridomas 300-1, 300-2, 300-3, 302-1 or 302-9. Particularly preferred are monoclonal antibodies corresponding to those produced by hybridomas 300-3 or 302-9.

The monoclonal antibodies of the invention may be used for the in vivo detection of antigen. The detectably labeled monoclonal

antibody is administered in a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled monoclonal antibody is administered in sufficient quantity to enable detection of the site having the antigens for which the monoclonal antibodies are specific. The concentration of detectably labeled monoclonal antibody which is administered should be sufficient that the binding to the site (for instance, a tumor) is detectable compared to the background signal. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best tumor-to-background signal ratio.

As a rule, the dosage of detectably labeled monoclonal antibody for diagnosis will vary depending on such factors as age, sex and extent of disease of the individual. The dosage of monoclonal antibody can vary from 0.01 $mg/m^2$ to 20 $mg/m^2$, preferably 0.1 $mg/m^2$ to 10 $mg/m^2$.

For diagnostic in vivo imaging, the type of detection instrument available is a major factor in selecting a given label. For example, if a radioisotope is chosen, it must have a type of decay which is detectable for a given type of instrument. Another important factor in selecting a radioisotope for in vivo diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Preferably, a radioisotope used for in vivo imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras. The monoclonal antibodies of the invention can also be labeled with a paramagnetic isotope for purposes of in vivo diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes or fluorescent labels are used for camera imaging and paramagnetic isotopes for NMR.

For in vivo diagnosis, labels may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind labels to immunoglobulins are the bifunctional chelating agents such as, diethylenetriaminepentaacetic acid (DTPA) and ethylenediamine-tetraacetic acid (EDTA) and similar molecules or agents such as biotin.

**Predicting Metastatic Potential of Melanomas**

It has been determined that metastatic melanomas and primary melanomas with metastatic potential secrete tenascin. Testing serum of patients whose skin lesions are suspected of being metastatic therefore provides supplemental evidence to aid in diagnosis. The preferred patients for testing are patients with Stage I primary melanoma, level 2, 3 or 4.

Blood samples are obtained from the patients and tested to determine the presence of tenascin and its level in the serum. The tenascin level may be determined using antibodies specific for tenascin and any immunoassay format used in the art. For example competitive immunoassay using tenascin which has been labelled with radioisotopes, derivatized with an enzyme or a fluorescent moiety or any similar label may be used. Alternatively, a sandwich-type assay may be used. Preferably, at least one of the antibodies used in the sandwich-type assay (either the immobilized "catching" antibody or the detecting antibody) is one of the monoclonal antibodies produced by hybridomas 300-1, 300-2, 300-3, 302-1 or 302-9, because these antibodies define four non-overlapping epitopes. Most preferably, both the catching and the detecting antibody will be antibodies of this invention. Generally, the antibodies are chosen to bind to two non-overlapping epitopes so that they may both bind to tenascin at the same time. In one embodiment, the detecting antibody itself is labelled for detection. Alternatively, an anti-mouse IgG carrying a detectable marker may be added to detect the immunocomplex.

Detection of high levels of tenascin in serum from patients with pigmented skin lesions, taken together with other positive indicia of metastasis, supports a diagnosis of metastatic melanoma.

Levels of tenascin which are at least about twice the levels found in normal serum are considered high. Alternatively, an increase over time in serum tenascin in a tumor patient may be taken to support the conclusion that the patient's tumor is becoming metastatic.

As contemplated by this invention serum tenascin may be determined by immunoassay carried out according to any of the conventional immunoassay formats, which are well known to those of ordinary skill in the art. In general, antibodies specific for human tenascin are contacted with a sample suspected of containing tenascin, and formation of an immune complex between the antibody and the antigen is detected by any of the detection means known in the art for immunoassay; for example, radioimmunoassay, enzyme-linked immunosorbent assay, complement fixation, nephelometric assay, immunodiffusion, immunoelectrophorectic assay, and the like. The various formats such as competitive immunoassay or sandwich immunoassay are well known in those skilled in the art. See, e.g., "Immunoassay: A Practical Guide" (D.W. Chan and M.T. Perlstein eds. 1987) the disclosure of which is incorporated herein by reference. Antibody specific for tenascin could be formulated into any conventional immunoassay format; e.g., homogeneous or heterogeneous, radioimmunoassay or ELISA. Polyclonal antibodies which contain an antibody species immunoreactive with an epitope on tenascin may be used as well as monoclonal antibodies reactive with tenascin.

**Glioma Therapy**

The ability to detect tenascin in frozen tissue sections can be used in surgical treatment of glioma. Surgical therapy for glioma is difficult because present methods for determination of the tumor boundary during surgery are imprecise. Therefore part of the tumor may inadvertently be left in the patient rather than being excised. This results in early remission. The present invention contemplates a novel method for detecting the boundary between tumor tissue and normal tissue while the surgical procedure is in progress. In the method of this invention, the presence of tenascin may be used as a marker for tumor tissue because glioma cells secrete tenascin. By

continually monitoring the tenascin level of the outermost excised tissue, preferably by means of a series of frozen sections probed with anti-tenascin antibodies which have been detectably labelled, all of the tenascin-containing tumor tissue may be excised, and the surgical procedure can be discontinued when the boundary with non-tenascin-containing normal tissue is reached.

Tenascin levels in the excised tissue are preferably monitored in frozen tissue sections. In a preferred procedure, a sample of the tissue may be fixed with cold ethanol in paraffin. Thin sections are deparaffinized and hydrated, then incubated with monoclonal antibodies specific for tenascin. Formation of immunocomplex indicates the presence of tenascin in the tissue.

The antibodies specific for tenascin can be used to detect tenascin epitopes in histological sections of tissue as well as in serum. Such antibodies are capable of binding to tenascin, and these antibodies permit detection when they are coupled with isotopes, conjugated antibodies, or other ligands. One can detect antibody binding to tissue sections by any detection means known in the art, for example, radioimmunoassay, enzyme-linked stains, immunofluorescent microscopy and the like. One example of a suitable material for autoradiography is $^{125}I$ conjugated to the antibodies.

A particularly useful stain employs peroxidase, hydrogen peroxide and a chromogenic substance such as aminoethyl carbazole. The peroxidase (a well known enzyme, available from many sources) can be coupled to the specific antibody or merely complexed to it via one or more antibodies. Other chromogenic substances and enzymes may also be used. Such techniques are well known in the art. Radio-labeled antibodies may be specific for the antigen or may be second antibodies immunoreactive with specific antibodies. Again, such techniques are well known. The precise technique by which the antigen is detected is not critical to the invention.

Example 1 Production of mAbs to a High $M_r$ Protein in Culture Supernatants of Melanoma Cells.

Cationic proteins were isolated from the conditioned media of two metastatic melanoma cell lines (WM 239A and WM 266-4) derived from the same patient (Herlyn, et al. (1985), J. Natl. Cancer Inst., 74:283-289) by adhesion to sulfated dextran (Nister, et al., (1984), Proc. Natl. Acad. Sci. USA, 81:926-930). The proteins were size fractionated by gel filtration chromatography on Sephadex G-200 (Pharmacia), and mice were immunized with the high $M_r$ fractions (>150,000 daltons) which were emulsified in adjuvant consisting of Lipid A of Salmonella Minnesota. Selection of hybridomas for binding to culture supernatants of melanoma cells, growth of hybridomas, cloning by limited dilutions, and purification of antibodies from ascitic fluid of hybridoma-bearing mice were done following standard protocols (Herlyn, et al. (1985), Cancer Res., 45:5670-5676).

**Binding Studies.** Binding of mAbs to antigens released by cultured cells was tested in solid phase RIA. Culture supernatants of confluent cultures were absorbed overnight at room temperature to polyvinylchloride microtiter wells. Non-specific binding of antibodies was blocked by absorption of wells with 2% BSA, and antigens were crosslinked before assay with 0.05% glutaraldehyde. Binding of mAbs was tested with $^{125}$I-labeled goat IgG antibinding $F(ab')_2$.

Additive competition assays (Herlyn, et al., Cancer Res., 40:3602-3609, 1980, incorporated herein by reference) were done to determine whether mixed MAbs would show additive binding in solid phase RIA which would indicate binding to different determinant. In this assay, saturating quantities of $^{125}$I-labeled secondary antibody were used.

Table 1 Binding of monoclonal antibody 300-3 to serum-free supernatants of cultured cell lines[a]

| Tissue Type | Cells | No. of cell lines tested | No. of supernatants of cell lines binding antibody in indirect radioimmunoassay | | | |
|---|---|---|---|---|---|---|
| | | | neg. (500 cpm) | weak (500-2,500 cpm) | moderate (2,500-5,000 cpm) | strong (>5,000 cpm) |
| Tumor | Metastatic Melanoma | 30 | 4 | 5 | 12 | 9 |
| | Primary Melanoma, advanced | 7 | 1 | 4 | 0 | 2 |
| | Primary Melanoma, early | 4 | 3 | 1 | 0 | 0 |
| | Glioma | 6 | 0 | 0 | 0 | 6 |
| | Carcinoma | 16 | 13 | 1 | 2 | 0 |
| | Lymphoma/Leukemia | 6 | 6 | 0 | 0 | 0 |
| Non-malignant lesion | Nevus | 8 | 6 | 2 | 0 | 0 |
| Normal | Melanocytes[b] | 15 | 12 | 3 | 0 | 0 |
| | Keratinocytes[c] | 4 | 4 | 0 | 0 | 0 |
| | Skin Fibroblasts | 9 | 0 | 4 | 4 | 1 |

a Confluent cultures grown in the presence of serum were washed twice and maintained for 3 to 5 days in the absence of serum. After removal of cellular debris, culture supernatants were tested in indirect solid phase radioimmunoassay for binding of monoclonal antibody.

b Melanocytes were grown in the presence of the phorbol ester TPA.

c Cultures were maintained prior to collection of supernatants in medium without pituitary extract.

**Binding Specificity.** Five mAbs (300-1, 300-2, 300-3, 302-1, 302-9), all of IgG1 isotype, from two independent fusion experiments were reactive with spent medium of melanoma cells. The mAbs did not bind to plasma or melanoma-derived fibronectin (as the other 34 mAbs from the same fusion experiments did) nor to collagen types I-IV, or laminin. Strong binding was seen to glioma-derived purified tenascin (Fig. 1). Control anti-tenascin mAb 81C6 showed similar binding. Direct and indirect competitive binding assays indicate the presence of at least 5 non-competing determinants that were detected with antibodies 300-1, 300-2, 300-3, 302-9, and 81C6. Binding of mAb 302-1 was inhibited by 302-9, but 302-1 was unable to inhibit binding of 302-9.

Cultures of metastatic melanomas, advanced primary melanomas, gliomas and normal fibroblasts secreted tenascin (Table 1). On the other hand, cultures of early primary melanomas, carcinomas (13 out of 16 cultures), lymphomas, leukemias, and normal keratinocytes did not secrete tenascin. Culture supernatants of 3 of 15 melanocyte cultures and 2 of 8 nevus cultures bound anti-tenascin mAbs at low but detectable levels. All five mAbs (300-1, 300-2, 300-3, 302-1, 302-9) showed similar binding patterns. Binding patterns to culture supernatants were the same as for mAb 81C6.

**Example 2 Secretion of Tenascin by Melanoma Cells.**

Quantitative double determinant RIAs with purified tenascin as the standard were done to determine tenascin secretion in spent medium of serum-free cultures. 96-well microtiter plates were precoated with mAb 300-3 (1 µg/ml). After incubation overnight and blocking of unspecific binding with BSA buffer (1% BSA + 0.05% Tween + 0.02% sodium azide), the wells were incubated with spent medium or purified tenascin for 4 h. Bound antigen was tested with $^{125}$I-labeled mAb 302-9 in solid phase RIA.

In quantitative double determinant RIAs with purified tenascin as standard, the level of secretion between different primary and metastatic melanoma cell lines varied and secretion ranged between 10 ng/ml and 910 ng/ml (Fig. 2). Metastatic melanomas secreted a median of 220 ng/ml tenascin compared to 80 ng/ml for primary

melanomas and 20 ng/ml for normal melanocytes. All of 34 melanoma cultures tested secreted fibronectin (results not shown). Fibronectin secretion was at similar levels to tenascin secretion in 13 of 16 cultures (81%). Collagen type IV and laminin secretion by 15 melanoma cultures, on the other hand, was dissimilar with levels often below detection. Tenascin could not be detected on the cell surface of melanomas (13 cultures tested) or gliomas (4 cultures tested).

Mixed hemadsorption assays for binding of mAbs to cell surfaces of cultured cells were done as described (Herlyn, et al. (1985), J. Immunol., 134:1300-1304).

**Example 3**

**Increase of Tenascin Secretion by TGF-β.** Melanocytes from two melanoma cell lines (WM 8, WM 852) were plated in 6-well culture plates in W 489 medium supplemented with EGF (5 ng/ml), insulin (5 µg/ml) and 2% FCS at 3 x $10^5$ cells/well. One day after plating, the medium was changed to serum-free medium with or without TGF-beta (1 ng/ml). After 3 days the conditioned medium was collected and assayed for the tenascin content by quantitative double determinant RIA.

Quantitative double determinant RIAs with purified tenascin as standard showed an increase of tenascin secretion in WM 852 cells from 125 ng/ml to 300 ng/ml. The supernatants of WM 8 cells, normally not secreting tenascin, did not contain a detectable tenascin concentration after incubation of cells with TGF-β.

Using serum-free cultures we were able to demonstrate induction of tenascin secretion for melanoma cells. This effect could only be observed in cells that normally produced tenascin, indicating that TGF-beta can upregulate but not initiate tenascin section.

**Example 4 Detection of Serum Antigen.**

Binding inhibition assays according to Herlyn, et al. (J. Clin. Immunol., 2:135-140, 1982) were done to determine the presence of soluble antigen in sera of patients. For this, antigen preparations were mixed with mAb adjusted to 50% of maximal binding. After

incubation overnight of 4°C reactivity of unbound antibody was tested in solid phase RIA.

The ability of tenascin in patients' sera to inhibit the binding of mAbs 302-9 and 300-3 to serum-free conditioned medium of metastatic melanoma cell line WM 9 was determined by binding inhibition assays (Table 2). Normal sera inhibited binding of both mAbs (means 17% ± 3.2 and 13.6% ± 4.1, respectively), suggesting the presence of circulating tenascin. In studies with mAbs 302-9 (Table 2), sera from melanoma patients with minimal or no apparent tumor burden (designated A, B, and C) and evident tumor burden (stage D) both showed elevated levels of circulating tenascin compared to controls (t values of 3.1 and 5.6, respectively). Furthermore, sera from melanoma patients with high tumor burden had more circulating tenascin than sera from patients with low tumor burden (t value of 3.5). Sera of patients with other cancers also had significantly elevated levels of tenascin compared to normal control sera (t value of 2.1). In studies with mAb 300-3, patients with advanced melanoma had significantly elevated circulating levels as compared to sera of normal donors (t value of 2.01), whereas patients with minimal or no evident melanoma and patients with other cancers did not differ significantly from normal donors (t values of 0.04 and 1.26, respectively).

Table 2. Binding of anti-tenascin antibodies to circulating tenascin in patients sera[a]

| Donor disease | No. of sera | Percent of inhibition of binding of MAb to target | |
|---|---|---|---|
| | | 302-9 | 300-3 |
| Melanoma A, B, C[b] | 32 | 22.8±1.8[c] | 13.5±5.7 |
| Melanoma D[d] | 65 | 31.5±3.4 | 21.3±5.1 |
| Other cancers[e] | 14 | 23.1±5.1 | 18.8±8.2 |
| Normal donors | 35 | 17±3.2 | 13.6±4.1 |

a Sera were diluted by mixing with equal quantity of monoclonal antibody which was prediluted to ~60% of maximal binding. Inhibition of binding of antibodies was tested in indirect radioimmunoassays on culture supernatants of melanoma WM 9.

b "Tumor burden" A-C indicate either no apparent disease after resection of primary (A, 2 sera), regional lymph node metastasis (B, 8 sera), or intact primary tumor before resection (C, 22 sera).

c Standard error of the mean.

d "Tumor burden" D indicates evident metastatic disease.

e Sera of patients with either breast carcinoma (2), pancreas carcinoma (2), colorectal carcinoma (2), renal cell carcinoma (2), or lymphoma (6).

DEPOSIT OF HYBRIDOMAS

The hybridomas producing the antibodies of this invention are: 300-1, 300-2, 300-3, 302-1, and 302-9, and they have been deposited under Accession Nos. _____, _____, _____, _____, and _____, respectively, at the American Type Culture Collection (ATCC), Rockville, Maryland, on September 5, 1991.

International Application No: PCT/ /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 5 line 12 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet [X]

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit | Accession Number

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet [X]

Hybridoma 300-1
For the designation of Denmark, the applicant request that the sample of the microorganism only be furnished to an expert until the application has been accepted or finally decided without having been accepted (Section 22(7)).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

Date of Deposit
Accession No. of Deposit

E [✓] This sheet was received with the international application when filed (to be checked by the receiving Office)

Virginia L. Tilley (Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau was

(Authorized Officer)

Form PCT/RO/134 (January 1985)

International Application No.

# M I C R O O R G A N I S M S

CONTINUATION B. ADDITIONAL INDICATIONS

In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC)

International Application No: PCT/ /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 5 line 12 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet [X]

Name of depositary institution

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Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

| Date of deposit | Accession Number |
|---|---|
| | |

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet [X]

Hybridoma 300-2
For the designation of Denmark, the applicant request that the sample of the microorganism only be furnished to an expert until the application has been accepted or finally decided without having been accepted (Section 22(7)).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

Date of Deposit
Accession No. of Deposit

E [X] This sheet was received with the international application when filed (to be checked by the receiving Office)

Virginia L. Ashby
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau is

(Authorized Officer)

(January 1985)

Form PCT/RO/134 (January 1981)

International Application No.

# M I C R O O R G A N I S M S

CONTINUATION B. ADDITIONAL INDICATIONS

In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC)

International Application No: PCT/ /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 5, line 12 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet [X]

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

| Date of deposit | Accession Number |
|---|---|
| | |

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet [X]

Hybridoma 300-3

For the designation of Denmark, the applicant request that the sample of the microorganism only be furnished to an expert until the application has been accepted or finally decided without having been accepted (Section 22(7)).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

Date of Deposit
Accession No. of Deposit

E [✓] This sheet was received with the international application when filed (to be checked by the receiving Office)

Virginia L. Utley (Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau was

(Authorized Officer)

Form PCT/RO/134 (January 1985)

International Application No.

# M I C R O O R G A N I S M S

CONTINUATION B. ADDITIONAL INDICATIONS

In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC)

International Application No: PCT/ /

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A. IDENTIFICATION OF DEPOSIT

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Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

| Date of deposit | Accession Number |
|---|---|
| | |

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

Hybridoma 302-1
For the designation of Denmark, the applicant request that the sample of the microorganism only be furnished to an expert until the application has been accepted or finally decided without having been accepted (Section 22(7)).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

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Accession No. of Deposit

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Form PCT/RO/134 (January 1985)

International Application No.

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International Application No: PCT/ /

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Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

| Date of deposit | Accession Number |
|---|---|
| | |

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet [X]

Hybridoma 302-9

For the designation of Denmark, the applicant request that the sample of the microorganism only be furnished to an expert until the application has been accepted or finally decided without having been accepted (Section 22(7)).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

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Accession No. of Deposit

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Virginia L Ulby (Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau is

(Authorized Officer)

(January 1985)

Form PCT RO/134 (January 1981)

International Application No.

# M I C R O O R G A N I S M S

CONTINUATION B. ADDITIONAL INDICATIONS

In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC)

## CLAIMS

1. A monoclonal antibody that specifically binds to an epitope on human tenascin, said epitope being specifically bound by a monoclonal antibody produced by one of the hybridomas 300-1, 300-2, 300-3, 302-1 or 302-9.

2. The monoclonal antibody of claim 1 wherein the antibody also comprises a detectable label.

3. A method for detecting human tenascin comprising

obtaining a sample of a human body fluid suspected of containing tenascin;

contacting the sample with a monoclonal antibody specific for tenascin; and

detecting immunocomplex.

4. The method of for detecting human tenascin, as recited in claim 3, wherein the monoclonal antibody in said contacting step corresponds to a monoclonal antibody produced by one of the hybridomas 300-1, 300-2, 300-3, 302-1 and 302-9.

5. In a method for determining metastatic potential of a pigmented skin lesion of a human patient, wherein the metastatic potential is judged by measuring the thickness of the lesion, measuring the mitotic rate within the lesion, and determining the number of tumor invading lymphocytes (TIL) within the lesion, the improvement comprising additionally determining the tenascin level in serum from the patient, an elevated tenascin level indicating increased metastatic potential.

6. In a method of therapy for glioma comprising surgical excision of the glioma tumor, the improvement comprising

preparing frozen sections from the boundary of the excised tissue;

contacting the frozen sections with a monoclonal antibody specific for tenascin; and

if immunocomplex formation is detected, excising additional tissue.

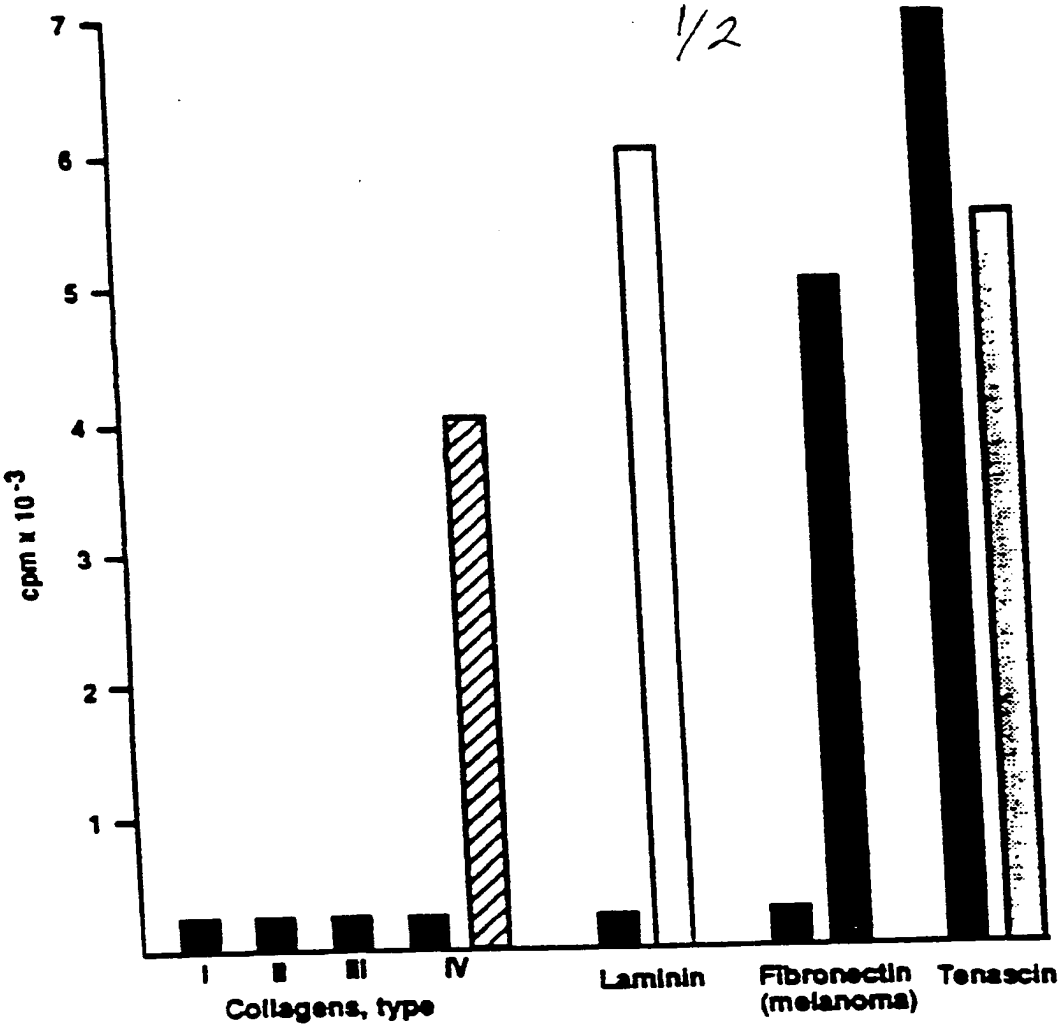
7
6
5
4
3
2
1
cpm x 10^-3
I
II
III
IV
Collagens, type
Laminin
Fibronectin
(melanoma)
Tenascin

Figure 1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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19 March 1992 (19.03.1992)

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(10) International Publication Number
WO 1992/004464 A3

(51) International Patent Classification[5]: C12P 21/08, G01N 33/574, 33/68, A61K 39/395

(21) International Application Number: PCT/US1991/006433

(22) International Filing Date: 5 September 1991 (05.09.1991)

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577,732 5 September 1990 (05.09.1990) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 577,732 (CIP)
Filed on 5 September 1990 (05.09.1990)

(71) Applicant *(for all designated States except US)*: **THE WISTAR INSTITUTE** [US/US]; Thirty-Sixth Street at Spruce, Philadelphia, PA 19104 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **HERLYN, Meenhard** [DE/US]; 1223 Knox Road, Wynwood, PA 19096 (US).

(74) Agents: **HOSCHEIT, Dale, H.** et al.; Banner, Birch, McKie & Beckett, 1001 G Street, N.W., 11th Floor, Washington, DC 20001 (US).

(81) Designated States *(national)*: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MC, MG, MN, MW, NL, NO, PL, RO, SD, SE, SU, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

**Published:**
— *with international search report*

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**Previous Correction:**
see PCT Gazette No. 13/1992 of 11 June 1992, Section II

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: MONOCLONAL ANTIBODIES AGAINST TENASCIN

(57) Abstract: This invention provides monoclonal antibodies that bind to four non-overlapping epitopes on human tenascin. This invention also provides an improved method for determining metastatic potential of melanomas, based on the discovery that metastatic melanomas secrete tenascin.

WO 1992/004464 A3

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/06433

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 21/08, G 01 N 33/574, 33/68, A 61 K 39/395

**II. FIELDS SEARCHED**

**Minimum Documentation Searched**[7]

| Classification System | Classification Symbols |
|---|---|
| IPC5 | G 01 N; A 61 K |

**Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched**[8]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**[9]

| Category * | Citation of Document,[11] with indication, where appropriate, of the relevant passages[12] | Relevant to Claim No.[13] |
|---|---|---|
| X | The Journal of Cell Biology, vol. 108, June 1989, Virginia A. Lightner et al.: "Tenascin/Hexabrachion in Human Skin: Biochemical Identification and Localization by Light and Electron Microscopy ", see page 2483 - page 2493 see page 2484, "Antibodies/Antigens" and "ELISA Assay for Tenascin" and page 2487, left column and fig 5 | 3 |
| Y | | 5 |
| | -- | |
| Y | Proc. Natl. Acad. Sci., vol. 84, July 1987, Eleanor J. Mackie et al.: "Tenascin is a stromal marker for epithelial malignancy in the mammary gland ", see page 4621 - page 4625 see "Inhibition ELISA", pages 4621-22 | 3 |
| | -- | |

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 14th January 1992 | 26. 02. 92 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | Weinberg |

Form PCT/ISA/210 (second sheet) (January 1985)

See notes on accompanying sheet

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 91/06433

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| Y | Int. J. Cancer: Supplement 4, 1989, Pier Giorgio Natali et al.: "Tenascin: A hexameric adhesive glycoprotein ", see page 66 - page 68 see page 67 | 1-5 |
| | -- | |
| X | Cell, vol. 59, October 1989, Jürg Spring et al.: "Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments ", see page 325 - page 334 | 1 |
| Y | | 1-5 |
| | -- | |
| Y | Journal of Neurochemistry, 1990, John R. Harper et al.: "Human neuroectoderm-derived cell line secretes fibronectin that shares the HNK-1/10C5 carbohydrate epitope with Neural cell adhesion molecules ", see page 395 - page 401 see page 395, page 397, right column - page 398, left column and page 400 | 1-5 |
| | -- | |
| A | CANCER RESEARCH, vol. 50, July 1990, Michael R. Zalutsky et al.: "Monoclonal antibody and F(ab')2 fragment delivery to tumor in patients with glioma: comparison of intracarotid and intravenous administration ", see page 4105 - page 4110 | 1 |
| | -- | |
| A | CANCER RESEARCH, vol. 47, August 1987, R.G. Blasberg et al.: "Regional localization of a glioma-associated antigen defined by monoclonal antibody 81C6 in Vivo: Kinetics and implications for diagnosis and therapy ", see page 4432 - page 4443 | 1 |
| | -- | |
| A | Cell, vol. 47, October 1986, Ruth Chiquet-Ehrismann et al.: "Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis ", see page 131 - page 139 | 1-5 |
| | -- | |



See notes on accompanying sheet

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 91/06433

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| Y | Dialog Informational Service, File 155, Medline 66-90/May, accession no. 06639065, medline accesssion no. 88284065, Inaguma Y et al: "Epithelial induction of stromal "tenascin" in the m ouse mammary gland from embryogenesis to carcino-genesis", & Dev Biol (UNITED STATES) Aug 1988, 128, (2) p245-55. | 1-5 |

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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 91/06433

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 6, because ~~they~~ it relate(s) to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional seach fees.